

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"EXPRESS MAIL LABEL NUMBER EM299799893US

DATE OF DEPOSIT: March 16, 1998

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER OF PATENTS, BOX PATENT APPLICATION, WASHINGTON, D.C. 20231

TAE KIM

(TYPED OR PRINTED NAME OF PERSON MAILING PAPER)

(SIGNATURE OF PERSON MAILING PAPER OR FEE)

A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

Method for Modulating Expression of  
Exogenous Genes in Mammalian Systems, and Products  
Related Thereto

by

Ronald M. Evans

David No

Enrique Saez

Sheets of Drawings: Six  
Docket No.: SALK1520-2  
Salk Ref. No.: S98001

Attorneys  
Gray Cary Ware & Freidenrich LLP  
4365 Executive Drive, Suite 1600  
San Diego, California 92121-2189

0304-2433-031633  
8537E0 88424050

Methods for Modulating Expression of Exogenous Genes  
in Mammalian Systems, and Products Related Thereto

RELATED APPLICATIONS

-2 ✓ This application is a continuation-in-part of  
5 United States Serial No. 08/974,530, filed November 19,  
<sup>abandoned</sup>  
1997, now ~~pending~~, which is, in turn, a continuation-in-  
part of United States Serial No. 08/628,830, filed April 5,  
-2 1996, now <sup>abandoned</sup>~~pending~~, the entire contents of both of which are  
hereby incorporated by reference herein.

10

FIELD OF THE INVENTION

The present invention relates to methods in the  
field of recombinant DNA technology, and products related  
thereto. More particularly, the invention relates to  
15 methods and products for modulating the expression of  
exogenous genes in mammalian systems.

BACKGROUND OF THE INVENTION

20

The steroid/thyroid hormone receptors comprise a  
superfamily of ligand-dependent transcription factors that  
play a crucial role in mediating changes in cell fate and  
function (Evans, R.M., *Science* **240**:889-895 (1988)). The  
receptors transduce extracellular hormonal signals to  
25 target genes that contain specific enhancer sequences  
referred to as hormone response elements (HREs) Evans,  
(1988); Green and Chambon, *Trends Genet.* **4**:309-314 (1988);  
Yamamoto, K.R., *Annu. Rev. Genet.* **19**:209-252 (1985)). Each  
receptor recognizes its own HRE, assuring that a distinct  
30 response is triggered by each hormonal signal. Together  
the collection of related transcription factors and their  
cognate response elements provides a unique opportunity to  
control gene expression.

09042438 031698

The DNA binding domain of each member of the steroid/thyroid hormone superfamily of receptors has 66-68 amino acids. Twenty of these, including nine cysteines, are conserved throughout the family. The modular structure of members of this receptor superfamily allows the exchange of homologous domains between receptors to create functional chimeras. This strategy was used to demonstrate that the DNA binding domain is solely responsible for the specific recognition of the HRE *in vivo* (Green and Chambon, 10 *Nature* 325:75-78 (1987); Giguère et al., *Nature* 330:624-629 (1987); Petkovich et al., *Nature* 330:444-450 (1987); Kumar et al., *Cell* 51:941-951 (1987); Umesono et al., *Nature* 336:262-265 (1988); Thompson and Evans, *Proc. Natl. Acad. Sci. U.S.A.* 86:3494-3498 (1989) and *in vitro* (Kumar and 15 Chambon, *Cell* 55:145-156 (1988)). By analogy with the proposed structure for *Xenopus* transcription factor IIIA (Miller et al., *EMBO J.* 4:1609-1614 (1985)), the invariant cysteines are thought to form two "zinc fingers" that mediate the DNA binding function (Hollenberg and Evans, 20 *Cell* 55:899-906 (1988)). Involvement of these cysteines in Zn(II) coordination is supported by extended X-ray absorption fine structure (Freedman et al., *Nature* 334:543-546 (1988)), and DNA binding by point mutagenesis experiments (Hollenberg and Evans, (1988)); Severne et al., 25 *EMBO J.* 7:2503-2508 (1988)).

The HREs are in fact structurally related but functionally distinct. The glucocorticoid receptor response element (GRE), estrogen receptor response element 30 (ERE), and thyroid hormone receptor response element (TRE) have been characterized in detail. These particular response elements have been found to have a palindromic pair of hexameric "half-sites" (Evans, (1988); Green and Chambon, (1988)). With optimized pseudo- or consensus 35 response elements, only two nucleotides per half-site differ between GRE and ERE (Klock et al., *Nature* 329:734-

034433 031698  
 869750 88424050

736 (1987)). On the other hand, EREs and TREs have identical half-sites but the number of nucleotide spacers between the two half sites is different (Glass et al., *Cell* 54:313-323 (1988)).

5

In contrast to response elements having the palindromic sequence motif, the following hormone receptors typically recognize response elements having two half-sites in a direct-repeat (DR) sequence motif: RXR, RAR, COUP-TF, 10 PPAR, and the like (see, e.g., Mangelsdorf et al., The Retinoids: Biology, Chemistry, and Medicine, 2nd Edition, Raven Press, Ltd., New York, 1994, Chapter 8). Thus at least three distinct means are used to achieve HRE 15 half-site; 2) nucleotide spacing between the two half-sites; and 3) the orientation of the half-sites to one another.

In insect systems, a pulse of the steroid hormone 20 ecdysone triggers metamorphosis in *Drosophila melanogaster* showing genomic effects, such as chromosomal puffing, within minutes of hormone addition. Mediating this response in insects is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product 25 of the *ultraspiracle* gene (USP) (Yao et al. (1993) *Nature* 366, 476-479; and Yao et al. (1992) *Cell* 71, 63-72). Responsiveness to an insect ecdysteroid can be recreated in cultured mammalian cells by co-transfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone 30 or the synthetic analog muristerone A.

In the field of genetic engineering, precise control of gene expression is an invaluable tool in studying, manipulating and controlling development and 35 other physiological processes. For example applications for regulated gene expression in mammalian systems include inducible gene targeting, overexpression of toxic and

031698-03424050

teratogenic genes, anti-sense RNA expression, and gene therapy (Jaenisch, R. (1988) *Science* **240**, 1468-1474). For cultured cells, glucocorticoids and other steroids have been used to induce the expression of a desired gene.

5

As another means for controlling gene expression in a mammalian system, an inducible tetracycline regulated system has been devised and utilized in transgenic mice, whereby gene activity is induced in the absence of the  
10 antibiotic and repressed in its presence (see, e.g, Gossen et al. (1992) *Proc. Natl. Acad. Sci.* **89**, 5547-5551; Gossen et al. (1993) *TIBS* **18**, 471-475; Furth et al. (1994) *Proc. Natl. Acad. Sci.* **91**, 9302-9306; and Shockett et al. (1995) *Proc. Natl. Acad. Sci.* **92**, 6522-6526). However,  
15 disadvantages of this system include the continuous treatment of tetracycline to repress expression and the slow clearance of antibiotic from bone which interferes with quick and precise induction. While this system has been improved by the recent identification of a mutant  
20 tetracycline repressor which acts conversely as an inducible activator, the pharmacokinetics of tetracycline may hinder its use during development when a precise and efficient "on-off" switch is essential (Gossen et al. (1995) *Science* **268**, 1766-1769).

25

Accordingly, there is a need in the art for improved methods to precisely modulate the expression of exogenous genes in mammalian subjects.

30

#### BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided various methods for modulating the expression of an exogenous gene in a mammalian subject. The invention  
35 method is useful in a wide variety of applications where inducible *in vivo* expression of an exogenous gene is desired, such as *in vivo* therapeutic methods for delivering

03042488 031698

recombinant proteins into a variety of cells within a patient.

Unlike prior art tetracycline based strategies, 5 transferring ecdysone responsiveness to mammalian cells takes advantage of a naturally evolved steroid inducible system. Advantages of ecdysteroid use include the lipophilic nature of the compounds (which provides efficient penetrance thereof into all tissues, including 10 the brain), short half-lives (which allow for precise and potent inductions), and favorable pharmacokinetics that prevent storage and expedite clearance.

In accordance with another embodiment of the 15 present invention, there are provided modified ecdysone receptors, which can be in the form of homodimeric species or heterodimeric species comprising at least one silent partner of the steroid/thyroid hormone superfamily of receptors, along with an invention modified ecdysone 20 receptor. Invention modified ecdysone receptors are useful, for example, in methods for modulating expression of an exogenous gene in a mammalian subject.

In accordance with additional embodiments of the 25 present invention, there are provided nucleic acids encoding invention modified ecdysone receptors, modified ecdysone receptor response elements, gene transfer vectors, recombinant cells, and transgenic animals containing nucleic acid encoding invention modified ecdysone receptor.

30

#### BRIEF DESCRIPTION OF THE FIGURES

Figures 1A - 1D show the optimization of ecdysone responsiveness using various combinations of USP 35 or RXR with different modified EcRs. In Figure 1A, the numerical values on both sides of the figure are on the same scale, with the GEcR/RXR value repeated for clarity.

Darkened and stripped bars represent reporter activity with no hormone or  $1\mu\text{M}$  muristerone A, respectively.

Figure 1B shows FXR and VpEcR activity on ecdysone response element (EcRE) and a hybrid ecdysone/glucocorticoid response element (E/GRE) responsive reporters. VpEcR, VgEcR, and control transfection without receptors were treated with  $1\mu\text{M}$  muristerone. FXR transfections were treated with  $50\mu\text{M}$  Juvenile Hormone III (Sigma). Darkened and stripped bars represent reporter activity with no hormone or  $1\mu\text{M}$  muristerone A/ $50\mu\text{M}$  Juvenile Hormone III, respectively.

Figure 1C shows that E/GRE and GRE are non-overlapping response elements. Darkened and stripped bars represent reporter activity with no hormone or  $1\mu\text{M}$  muristerone A/ $1\mu\text{M}$  dexamethasone, respectively.

Figure 1D shows a schematic diagram of modified ecdysone receptors. GEcR is a chimeric receptor containing the N-terminal transactivation domain of GR and the DNA- and ligand-binding domains of EcR. VpEcR is an N-terminal truncation of EcR, wherein the activation domain of Vp16 is fused thereto at the amino terminus thereof. VgEcR is identical to VpEcR except for the following point mutations in the P box of the DNA binding domain: E282G, G283S, and G286V. Vp16-EcR-B1 is a fusion of full length EcR with the activation domain of Vp16, wherein the activation domain of Vp16 is fused thereto at the carboxy terminus thereof. VgEcR-B1 is identical to Vp16-EcR-B1 except for the same point mutations in the P box of the DNA binding domain as described above. In the Figure, DBD=DNA binding domain and LBD=ligand binding domain.

Figure 2 shows a schematic diagram of an invention ecdysone inducible gene expression system. After

09042488 031698  
SECRET

expression of RXR and a modified EcR, the two receptors can heterodimerize and transactivate the ecdysone response element-containing promoter in the presence of hormone. The ecdysone response elements are placed upstream of a 5 minimal promoter (i.e., an enhancerless promoter) which can drive the expression of any exogenous cDNA.

Figure 3A shows a dose-dependent activation of N13 cells with muristerone. N13 cells were grown with 10 varying concentrations of muristerone for 36 hours and then assayed for  $\beta$ -galactosidase activity (open squares) by standard ONPG assay or for luciferase activity (closed circles). Figure 3B shows the time-course of luciferase activity of N13 cells treated with hormone. N13 cells were 15 grown in separate wells in the presence of  $1\mu\text{M}$  muristerone, harvested at varying times, and assayed for luciferase activity as described in Example 3.

Figure 4 shows muristerone activity in mice as 20 described in Example 4.

Figure 5 compares the dose-dependent activation of N13 cells with muristerone (X) and ponasterone A (open circles).

25

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided methods for modulating the expression of an 30 exogenous gene in a mammalian subject containing:

- (i) a DNA construct comprising said exogenous gene under the control of an ecdysone response element; and
- (ii) a modified ecdysone receptor which, in the 35 presence of a ligand therefor, and optionally in the further presence of a

09042488 031698

receptor capable of acting as a silent partner therefor, binds to said ecdysone response element;

said method comprising administering to said  
5 subject an effective amount of a ligand for said modified ecdysone receptor; wherein said ligand is not normally present in the cells of said subject; and wherein said ligand is not toxic to said subject.

10 Thus, in accordance with the present invention the insect molting hormone, ecdysone (as well as analogs and mimics thereof), is advantageously employed as a regulated inducer of gene expression in mammalian systems, i.e., background levels of expression are substantially  
15 zero in the absence of conditions required for induction. In a presently preferred aspect of the invention, promoters containing a novel modified ecdysone response element are employed in conjunction with an invention modified ecdysone receptor (preferably having an altered DNA binding  
20 specificity) to provide an extremely powerful and specific inducible mammalian expression system. The low basal activity of the invention expression system is advantageously suitable for the expression of transcription factors and toxic genes. The excellent dose response and  
25 induction rate characteristics of the invention inducible expression system allow for precise control of both the degree and duration of induction of a desired gene.

Since the invention method provides for regulated  
30 gene expression by an exogenous non-mammalian inducer, it can be advantageously employed in a variety of *in vivo* and *in vitro* mammalian expression systems. For example, inducible expression of cre recombinase in transgenic mammals, in accordance with invention methods, would enable  
35 those of skill in the art to accomplish temporally specific inducible gene targeting of the adult or the developing embryo (O'Gorman et al. (1991) *Science* **251**, 1351-1355).

09042488 031698

As employed herein, the terms "modulate" and "modulating" refer to the ability of a given ligand/receptor complex to effect transactivation of transcription of an exogenous gene, relative to such ability of said receptor in the absence of ligand. The actual effect of complex formation on the transactivation activity of a receptor will vary depending on the specific receptor species which are part of the ligand/receptor complex, and on the response element with which the ligand/receptor complex interacts.

As used herein, when referring to genes, the phrase "exogenous to said mammalian subject" or simply "exogenous" refers to any gene wherein the gene product is not naturally expressed in the particular cell where expression is desired. For example, exogenous genes can be either natural or synthetic wild type genes and therapeutic genes, which are introduced into the subject in the form of DNA or RNA. The gene of interest can be introduced into target cells (for *in vitro* applications), or the gene of interest can be introduced directly into a subject, or indirectly introduced by the transfer of transformed cells into a subject.

25

"Wild type" genes are those that are native to cells of a particular type. Such genes may be undesirably overexpressed, or may not be expressed in biologically significant levels. Thus, for example, while a synthetic or natural gene coding for human insulin would be exogenous genetic material to a yeast cell (since yeast cells do not naturally contain insulin genes), a human insulin gene inserted into a human skin fibroblast cell would be a wild type gene with respect to that cell since human skin fibroblasts contain genetic material encoding human insulin, although human skin fibroblasts do not express human insulin in biologically significant levels.

03042433 031633  
359750 33424050

Wild type genes contemplated for use in the practice of the present invention include genes which encode a gene product:

- 5           the substantial absence of which leads to the occurrence of a non-normal state in said subject; or
- a substantial excess of which leads to the occurrence of a non-normal state in said subject;
- 10          and the like.

As employed herein, the phrase "therapeutic gene" refers to a gene which imparts a beneficial function to the host cell in which such gene is expressed. Therapeutic  
15 genes are those that are not naturally found in host cells.

For example, a synthetic or natural gene coding for wild type human insulin would be therapeutic when inserted into a skin fibroblast cell so as to be expressed in a human host, where the human host is not otherwise capable of  
20 expressing functionally active human insulin in biologically significant levels. In accordance with the methods described herein, therapeutic genes are expressed at a level that provides a therapeutically effective amount of the corresponding therapeutic protein.

25           Therapeutic genes contemplated for use in the practice of the present invention include genes which encode a gene product:

- 30          which is toxic to the cells in which it is expressed; or
- which imparts a beneficial property to the host subject (e.g., disease resistance, etc);
- and the like.

35           Numerous genomic and cDNA nucleic acid sequences coding for a variety of proteins are well known in the art.

Exogenous genetic material useful in the practice of the

09042488-031598

present invention include genes that encode biologically active proteins of interest, such as, e.g., secretory proteins that can be released from said cell; enzymes that can metabolize a substrate from a toxic substance to a non-toxic substance, or from an inactive substance to a useful substance; regulatory proteins; cell surface receptors; and the like. Useful genes include genes that encode blood clotting factors such as human factors VIII and IX; genes that encode hormones such as insulin, parathyroid hormone, luteinizing hormone releasing factor (LHRH), alpha and beta seminal inhibins, and human growth hormone; genes that encode proteins such as enzymes, the absence of which leads to the occurrence of an abnormal state; genes encoding cytokines or lymphokines such as interferons, granulocytic macrophage colony stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-1), tumor necrosis factor (TNF), and erythropoietin (EPO); genes encoding inhibitor substances such as  $\alpha_1$ -antitrypsin; genes encoding substances that function as drugs, e.g., genes encoding the diphtheria and cholera toxins; and the like.

Typically, nucleic acid sequence information for a desired protein can be located in one of many public access databases, e.g., GENBANK, EMBL, Swiss-Prot, and PIR, or in many biology related journal publications. Thus, those of skill in the art have access to nucleic acid sequence information for virtually all known genes. Those of skill in the art can either obtain the corresponding nucleic acid molecule directly from a public depository or the institution that published the sequence. Optionally, once the nucleic acid sequence encoding a desired protein has been ascertained, the skilled artisan can employ routine methods, e.g., polymerase chain reaction (PCR) amplification, to isolate the desired nucleic acid molecule from the appropriate nucleic acid library. Thus, all known nucleic acids encoding proteins of interest are available for use in the methods and products described herein.

As used herein, the terms "mammal" and "mammalian" refer to humans; domesticated animals, e.g., rats, mice, rabbits, canines, felines, and the like; farm animals, e.g., chickens, bovine, porcine and ovine, and the like; and animals of zoological interest, e.g., monkeys and baboons, and the like.

Modified ecdysone receptors contemplated for use in the practice of the present invention comprise:

a ligand binding domain capable of binding an ecdysteroid;

a DNA-binding domain obtained from a DNA-binding protein; and

an activation domain of a transcription factor,

wherein at least one of said DNA-binding domain or said activation domain is not obtained from a native ecdysone receptor,

with the proviso that when said activation domain is derived from a glucocorticoid receptor, said DNA-binding domain is not derived from a glucocorticoid receptor or an *E. coli* LexA protein. In accordance with the present invention, modified ecdysone receptors function in expression systems, preferably mammalian, to transactivate gene expression from transcription regulatory regions having ecdysone response elements. Preferably, in order to minimize induction of undesired gene expression, modified ecdysone receptors of the invention will have substantially no constitutive activity in mammalian cells.

Ligand binding domains capable of binding an ecdysteroid, as contemplated for use in the preparation of invention modified ecdysone receptors are typically derived from the carboxy-terminal portion of native ecdysone receptor and are able to bind ecdysteroids (Koelle et al., *Cell*, 67:59-77, 1991; and Christopherson et al., *PNAS, USA*,

09042488 031698

89:6314-6318, 1992). Ligand binding domains capable of binding an ecdysteroid can be functionally located in either orientation and at various positions within the modified ecdysone receptor of the invention. For example, 5 the ligand binding domain capable of binding an ecdysteroid can be positioned at either the amino or carboxy terminus of the modified receptor, or therebetween. In a preferred embodiment of the present invention, the ligand binding domain capable of binding an ecdysteroid is positioned at 10 the carboxy terminus of the modified receptor (see Figure 1D).

DNA-binding domains contemplated for use in the preparation of invention modified ecdysone receptors are 15 typically obtained from DNA-binding proteins (e.g., transcription factors). The term "DNA-binding domain" is understood in the art to refer to an amino acid sequence that is able to bind to DNA. As used herein, the term "DNA-binding domain" encompasses a minimal peptide sequence 20 of a DNA-binding protein, up to the entire length of a DNA-binding protein, so long as the DNA-binding domain functions to associate with a particular response element.

Such DNA-binding domains are known to function 25 heterologously in combination with other functional protein domains by maintaining the ability to bind the natural DNA recognition sequence (see, e.g., Brent and Ptashne, 1985, Cell, 43:729-736). For example, hormone receptors are known to have interchangeable DNA-binding domains that 30 function in chimeric proteins (see, e.g., U.S. Patent No. 4,981,784; and Evans, R., 1988, Science, 240:889-895). Thus, similar to the ligand binding domain of invention modified ecdysone receptor, the DNA-binding domain can be positioned at either the carboxy terminus or the amino 35 terminus, or the DNA-binding domain can be positioned between the ligand binding domain and the activation domain. In preferred embodiments of the present invention,

09042463 1031693  
35972050

the DNA-binding domain is positioned internally between the ligand binding domain and the activation domain.

"DNA-binding protein(s)" contemplated for use herein belong to the well-known class of proteins that are able to directly bind DNA and facilitate initiation or repression of transcription. Exemplary DNA-binding proteins contemplated for use herein include transcription control proteins (e.g., transcription factors and the like; Conaway and Conaway, 1994, "Transcription Mechanisms and Regulation", Raven Press Series on Molecular and Cellular Biology, Vol. 3, Raven Press, Ltd., New York, NY).

Transcription factors contemplated for use herein as a source of such DNA binding domains include, e.g., homeobox proteins, zinc finger proteins, hormone receptors, helix-turn-helix proteins, helix-loop-helix proteins, basic-Zip proteins (bZip),  $\beta$ -ribbon factors, and the like.

See, for example, Harrison, S., "A Structural Taxonomy of DNA-binding Domains," Nature, 353:715-719. Homeobox DNA-binding proteins suitable for use herein include, for example, HOX, STF-1 (Leonard et al., 1993, Mol. Endo., 7:1275-1283), Antp, Mat  $\alpha$ -2, INV, and the like. See, also, Scott et al. (1989), Biochem. Biophys. Acta, 989:25-48. It has been found that a fragment of 76 amino acids (corresponding to amino acids 140-215 described in Leonard et al., 1993, Mol. Endo., 7:1275-1283) containing the STF-1 homeodomain binds DNA as tightly as wild-type STF-1. Suitable zinc finger DNA-binding proteins for use herein include Zif268, GLI, XFin, and the like. See also, Klug and Rhodes (1987), Trends Biochem. Sci., 12:464; Jacobs and Michaels (1990), New Biol., 2:583; and Jacobs (1992), EMBO J., 11:4507-4517.

Preferably, the DNA-binding domain used herein is obtained from a member of the steroid/thyroid hormone

09042488 "031698  
96972060

superfamily of receptors. As used herein, the phrase "member(s) of the steroid/thyroid hormone superfamily of receptors" (also known as "nuclear receptors" or "intracellular receptors") refers to hormone binding proteins that operate as ligand-dependent transcription factors, including identified members of the steroid/thyroid hormone superfamily of receptors for which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors").

10

Exemplary members of the steroid/thyroid hormone superfamily of receptors (including the various isoforms thereof) include steroid receptors such as glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), vitamin D<sub>3</sub> receptor (VDR), and the like; plus retinoid receptors, such as the various isoforms of retinoic acid receptor (e.g., RAR $\alpha$ , RAR $\beta$ , or RAR $\gamma$ ), the various isoforms of retinoid X receptor (e.g., RXR $\alpha$ , RXR $\beta$ , or RXR $\gamma$ ), and the like (see, e.g., U.S. Patent Nos. 4,981,784; 5,171,671; and 5,071,773); thyroid receptors (TR), such as TR $\alpha$ , TR $\beta$ , and the like; insect derived receptors such as the ecdysone receptor, and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove, including the various isoforms thereof. Examples of orphan receptors contemplated for use herein as a source of DNA binding domain include HNF4 (see, for example, Sladek et al., in Genes & Development 4: 2353-2365 (1990)), the COUP family of receptors (see, for example, Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), and Wang et al., in Nature 340: 163-166 (1989)), COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), various isoforms of peroxisome

00042438 031698  
 00000000 00000000

proliferator-activated receptors (PPARs; see, for example, Issemann and Green, supra), the insect derived knirps and knirps-related receptors, and the like.

5           The DNA-binding domains of all members of the steroid/thyroid hormone superfamily of receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines. A member of the superfamily can  
10 be characterized as a protein which contains these 20 invariant amino acid residues. The highly conserved amino acids of the DNA-binding domain of members of the superfamily are as follows:

15           Cys - X - X - Cys - X - X - Asp\* - X -  
Ala\* - X - Gly\* - X - Tyr\* - X - X -  
X - X - Cys - X - X - Cys - Lys\* - X -  
Phe - Phe - X - Arg\* - X - X - X - X -  
X - X - X - X - X - (X - X -) Cys - X  
20           -X - X - X - X - (X - X - X -) Cys - X  
-X - X - Lys - X - X - Arg - X - X -  
Cys - X - X - Cys - Arg\* - X - X -  
Lys\* - Cys - X - X - X - Gly\* - Met  
(SEQ ID NO:1);

25           wherein X designates non-conserved amino acids within the DNA-binding domain; an asterisk denotes the amino acid residues which are almost universally conserved, but for which variations have been found in some identified hormone  
30 receptors; and the residues enclosed in parenthesis are optional residues (thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues).

35           Modification of existing DNA-binding domains to recognize new target recognition sequences is also contemplated herein. For example, in accordance with the

09042488-031698  
SECRETED 88424060

present invention, it has been found that the modification of the "P-box" sequence of DNA-binding domains of members of the steroid/thyroid hormone superfamily of receptors offers unique advantages not present in other chimeric hormone receptors. For example, the modification of a P-box amino acid sequence to preferentially bind to a different hormone response element half-site than the naturally occurring P-box amino acid sequence can reduce undesired background levels of gene expression. Thus, invention receptors and methods provide the advantage of increasing the selectivity of exogenous gene expression in a particular subject.

As used herein, the phrase "P-box amino acid sequence" refers to the proximal element region in a DNA-binding domain of a hormone receptor that typically occurs at the junction of the first zinc finger and the linker region, e.g., at about amino acids 19-23 of the DNA-binding domain (i.e., amino acids 19-23 of SEQ ID NO:1; see, e.g., Umesono et al. (1989), Cell, 57:1139-1146, Figure 2). Umesono et al. (1989), supra, in Table 1, describe various naturally occurring P-box amino acid sequences for a variety of hormone receptor DNA-binding domains.

In one embodiment of the present invention, the P-box sequence of a hormone receptor DNA-binding domain is modified to have a P-box amino acid sequence that differs from the naturally occurring P-box amino acid sequence. In a preferred embodiment of the present invention, the modified P-box amino acid sequence differs from the naturally occurring P-box amino acid sequence by 3 amino acids.

Preferably, the P-box amino acid sequence is modified so that only the half-site nucleotide sequence recognized by the DNA-binding domain is changed while not altering the spacing between the two half-sites recognized

03042433 031698

by the DNA-binding domain. For example, when the DNA-binding domain of the ecdysone receptor is employed in an invention modified ecdysone receptor, the P-box can be modified from the amino acid sequence EGCKG (SEQ ID NO:2; 5 which recognizes the half-site -AGGTCA-) to the amino acid sequence GSCKV (SEQ ID NO:3; which recognizes the half-site sequence -AGAACA-). In a presently preferred embodiment, when the DNA-binding domain of invention modified ecdysone receptor is derived from ecdysone receptor, the P-box amino 10 acid sequence is modified to GSCKV (SEQ ID NO:3).

It has also been found that *in vitro* evolution methods can be applied to modify and improve existing DNA-binding domains (see, e.g., Devlin et al., 1990, Science, 15 249:404-406; and Scott and Smith, 1990, Science, 249:386-390).

Activation domains contemplated for use in the preparation of invention modified ecdysone receptor are 20 typically derived from transcription factors and comprise a contiguous sequence of amino acids that functions to activate gene expression when associated with a suitable DNA-binding domain and a suitable ligand binding domain. As with the ligand and DNA-binding domains employed for the 25 preparation of invention modified ecdysone receptors, the activation domain can be positioned at the carboxy terminus, the amino terminus or between the ligand binding domain and the DNA binding domain. In preferred embodiments of present invention, the activation domain is 30 positioned at the amino terminus or the carboxy terminus of the modified ecdysone receptor.

Suitable activation domains can be obtained from a variety of sources, e.g., from the N-terminal region of a 35 member of the steroid/thyroid hormone superfamily of receptors, from a transcription factor activation domain, such as, for example, VP16 or GAL4 activation domains, and

09042483 031698

the like. The presently most preferred activation domain contemplated for use in the practice of the present invention is obtained from the N-terminal region of the VP16 protein.

5

The presently most preferred modified ecdysone receptors contemplated for use herein are VgEcR (SEQ ID NO:5), VpEcR (SEQ ID NO:7), GEcR (SEQ ID NO:9), Vp16-EcR-B1 or VgEcR-B1, with VgEcR (SEQ ID NO:5) and VgEcR-B1 being especially preferred. The preparation of several of these modified ecdysone receptors is set forth hereinafter in Example 1. See also Figure 1D. Those modified receptors for which explicit methods of preparation is not provided herein can readily be made using the methodology set forth herein in combination with standard methodology well known to those of skill in the art.

Invention modified ecdysone receptor proteins can be produced by expressing nucleic acid constructs encoding the chimeric proteins in suitable host cells as described in Example 1. Recombinant methods of producing desired proteins by introducing an expression construct into appropriate host cells are well-known in the art. Modified ecdysone receptors of the invention can be introduced into a particular subject by direct introduction of the proteins themselves, by introducing DNA construct(s) encoding the receptor into the subject, or into cells obtained from the subject (wherein the cells are transformed and subsequently returned to the subject).

25  
30

In a preferred embodiment, invention modified ecdysone receptors are expressed under the control of a tissue specific promoter. As readily understood by those of skill in the art, the term "tissue specific" refers to the substantially exclusive initiation of transcription in the tissue from which a particular promoter drives expression of a given gene.

In accordance with one aspect of the present invention, invention modified ecdysone receptors are present in the form of heterodimeric species comprising an invention modified ecdysone receptor and at least one silent partner of the steroid/thyroid hormone superfamily of receptors. Preferably, the silent partner is a mammalian-derived receptor, with RXR being especially preferred.

10

Silent partners contemplated herein are members of the steroid/thyroid hormone superfamily of receptors which are capable of forming heterodimeric species with the invention modified ecdysone receptor, wherein the silent partner does not directly participate in binding ligand (i.e., only the modified ecdysone receptor co-partner of the heterodimer binds ligand). The silent partner can either be endogenous to the cells of the subject or can be provided to the subject by introducing DNA construct(s) encoding receptor into the subject. A preferred silent partner for use herein is RXR. In a particular embodiment of the invention methods, exogenous RXR is provided to said mammalian subject.

25

The formation of heterodimeric receptor(s) can modulate the ability of member(s) of the steroid/thyroid hormone superfamily of receptors to trans-activate transcription of genes maintained under expression control in the presence of ligand for said receptor. For example, formation of a heterodimer of the modified ecdysone receptor with another mammalian hormone receptor promotes the ability of the modified ecdysone receptor to induce trans-activation activity in the presence of an ecdysone response element.

35

In accordance with another aspect of the present invention, invention modified ecdysone receptors are

031693-031693

present in the form of homodimeric species comprising a plurality (i.e., at least two) invention modified ecdysone receptors.

5           Ligands contemplated for use herein are compounds which, inside a cell, bind to invention modified ecdysone receptors, thereby creating a ligand/receptor complex, which in turn can bind to an appropriate response element.

10           The terms "ecdysone", "ecdysteroid", "ecdysone-analogs", and "ecdysone mimics" as interchangeably used herein, are employed herein in the generic sense (in accordance with common usage in the art), referring to a family of ligands with the appropriate binding and transactivation activity (see, for example, Cherbas et al., in *Biosynthesis, metabolism and mode of action of invertebrate hormones* (ed. J. Hoffmann and M. Porchet), p. 305-322; Springer-Verlag, Berlin). An ecdysone, therefore, is a steroid, steroid-like or non-steroidal compound which acts to modulate gene transcription for a gene maintained under the control of a  
15           suitable response element, as described herein.  
20

          20-Hydroxy-ecdysone (also known as  $\beta$ -ecdysone) is the major naturally occurring ecdysone. Unsubstituted ecdysone (also known as  $\alpha$ -ecdysone) is converted in  
25           peripheral tissues to  $\beta$ -ecdysone. Analogs of the naturally occurring ecdysones are also contemplated within the scope of the present invention. Examples of such analogs, commonly referred to as ecdysteroids, include ponasterone A, ponasterone B, ponasterone C, ponasterone D,  
30           26-iodoponasterone A, muristerone A, inokosterone, 26-mesylinokosterone, sidasterone, buterosterone, ajugasterone, makisterone, cyasterone, sengosterone, and the like. Since it has been previously reported that the above-described ecdysones are neither toxic, teratogenic,  
35           nor known to affect mammalian physiology, they are ideal

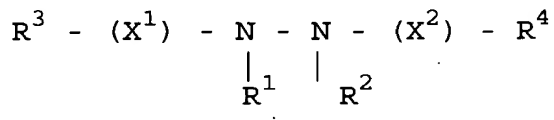
09042488-031698

candidates for use as inducers in cultured cells and transgenic mammals according to the invention methods.

Additional compounds contemplated for use herein  
 5 are mimics of the naturally occurring ecdysones, i.e.,  
 synthetic organic compounds which have binding and  
 transactivation activities characteristic of the naturally  
 occurring ecdysones. Examples of such compounds, referred  
 to herein as ecdysone mimics, include 1,2-diacyl hydrazines  
 10 (e.g., those described in U.S. Patent Nos. 5,424,333 and  
 5,354,762, the entire contents of each of which are hereby  
 incorporated by reference herein), N'-substituted-N,N'-di-  
 substituted hydrazines (e.g., those described in U.S.  
 Patent No. 5,117,057, the entire contents of which are  
 15 hereby incorporated by reference herein), dibenzoylalkyl  
 cyanohydrazines (e.g., those described in European  
 Application No. 461,809, the entire contents of which are  
 hereby incorporated by reference herein), N-substituted-N-  
 alkyl-N,N'-diaroyl hydrazines (e.g., those described in  
 20 U.S. Patent No. 5,225,443, the entire contents of which are  
 hereby incorporated by reference herein), N-substituted-N-  
 acyl-N-alkyl, carbonyl hydrazines (e.g., those described in  
 European Application No. 234,944, the entire contents of  
 which are hereby incorporated by reference herein),  
 25 N-aroyl-N'-alkyl-N'-aroyl hydrazines (e.g., those described  
 in U.S. Patent No. 4,985,461, the entire contents of which  
 are hereby incorporated by reference herein), and the like.

Compounds of specific interest are those having the  
 formula:

30



35 wherein:

$R^1$  is optionally hydrogen, lower alkyl or substituted lower alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, aryl or substituted aryl, heteroaryl or substituted heteroaryl, and the like.  $R^1$  is not present when  $X^1$  is part of a carbon-nitrogen double bond linking  $R^3$  to the hydrazino group.

$R^2$  is optionally hydrogen, alkyl or substituted alkyl, cyclohexyl or substituted cyclohexyl, and the like.  $R^2$  is not present when  $X^2$  is part of a carbon-nitrogen double bond linking  $R^4$  to the hydrazino group.

$R^3$  and  $R^4$  are independently part of an appropriately substituted carbon-nitrogen double bond which links  $R^3$  and/or  $R^4$  to the hydrazino linkage, or  $R^3$  and  $R^4$  are independently aryl or substituted aryl, heteroaryl or substituted heteroaryl, provided, however, that when two adjacent positions on the aryl or heteroaryl moieties are substituted with alkoxy, thioalkyl, alkylamino, or dialkylamino groups, these groups may be joined to form a 5- or 6- membered heterocyclic ring system, or  $R^3$  and  $R^4$  are independently heterocyclic or substituted heterocyclic, cycloalkyl or substituted cycloalkyl, and the like.

$X^1$  and  $X^2$  are independently  $-C(O)-$ ,  $-C(S)-$ ,  $-C(NR_2)-$ ,  $-C(=CN)NH-$ ,  $-C(O)O-$ ,  $-C(O)NH-$ ,  $-C(O)NHSO_2-$ ,  $-CH_2-$ ,  $-SO_2-$ ,  $-P(O)CH_3-$ , and the like, as well as an appropriate substituted carbon-nitrogen double bond which links  $R^3$  and/or  $R^4$  to the hydrazino linkage.

As employed herein, "alkyl" refers to alkyl groups having in the range of 1 up to 8 carbon atoms; "lower alkyl" refers to alkyl groups having in the range of 1 up to 4 carbon atoms; and "substituted alkyl" or "substituted lower alkyl" comprises alkyl (or lower alkyl) groups further bearing one or more substituents selected from halogen, cyano, nitro, hydroxy, alkoxy ( $-OR$ ),

thioalkyl (-SR), -NR<sub>2</sub>, -NRC(O)R, -OC(O)R, -C(O)OR, -C(O)NR<sub>2</sub>, -C(O)R, wherein each R is independently hydrogen or lower alkyl, and the like.

5           As employed herein, "cycloalkyl" refers to cyclic ring-containing groups containing in the range of about 5 up to 8 carbon atoms, and "substituted cycloalkyl" refers to cycloalkyl groups further bearing one or more substituents as set forth above, as well as lower alkyl.

10           As employed herein, "heterocyclic" refers to cyclic (i.e., ring-containing) groups containing one or more (up to four) heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and having in the range of 2  
15 up to 5 nuclear carbon atoms and "substituted heterocyclic" refers to heterocyclic groups further bearing one or more substituents as set forth above, as well as lower alkyl.

As employed herein, "alkenyl" refers to straight  
20 or branched chain hydrocarbyl groups having at least one carbon-carbon double bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkenyl" refers to alkenyl groups further bearing one or more substituents as set forth above.

25           As employed herein, "alkynyl" refers to straight or branched chain hydrocarbyl groups having at least one carbon-carbon triple bond, and having in the range of about 2 up to 12 carbon atoms, and "substituted alkynyl" refers  
30 to alkynyl groups further bearing one or more substituents as set forth above.

As employed herein, "aryl" refers to aromatic groups having in the range of 6 up to 14 carbon atoms and  
35 "substituted aryl" refers to aryl groups further bearing one or more substituents as set forth above, as well as lower alkyl.

090424188-031698

As employed herein, "heteroaryl" refers to aromatic groups containing one or more heteroatoms (e.g., N, O, S, or the like) as part of the ring structure, and 5 having in the range of 3 up to 14 carbon atoms and "substituted heteroaryl" refers to heteroaryl groups further bearing one or more substituents as set forth above.

10 Presently preferred ecdysone mimics contemplated for use herein include compounds wherein  $R^1$  is hydrogen;  $R^2$  is an alkyl group possessing considerable bulk (such as, for example, alkyl groups containing a tertiary carbon center, e.g.,  $-C(R'')_3$ , wherein each  $R''$  is methyl or 15 greater). Examples of alkyl groups having sufficient bulk for use herein include tert-butyl, sec-butyl, isopropyl, isobutyl, cyclohexyl, cyclopentyl, dicyclopropylmethyl, (cyclohexyl)ethyl, and the like);  $X^1$  and  $X^2$  are both  $-C(O)-$ ;  $R^3$  is phenyl, substituted phenyl (with hydroxy, alkoxy, 20 halo and/or substituted amino substituents being preferred, with 3,4-disubstitution pattern being especially preferred), heterocyclic (e.g., pyridyl or pyrimidine) or substituted heterocyclic (with halo, alkyl, thioalkyl, hydroxy, alkoxy, and/or amino substituents being 25 preferred); and  $R^4$  is phenyl or substituted phenyl, heteroaryl or substituted heteroaryl or a bulky alkyl or cycloalkyl group.

Especially preferred ecdysone mimics contemplated 30 for use herein include  $N'-(3,5\text{-dimethylbenzoyl})-N-(4\text{-ethylbenzoyl})-N'-(\text{tert-butyl})$  hydrazine,  $N,N'\text{-dibenzoyl}-N'-(\text{tert-butyl})$  hydrazine,  $N'-(3,5\text{-dimethylbenzoyl})-N-(4\text{-ethylbenzyl})-N'-(\text{tert-butyl})$  hydrazine,  $N'-(3,5\text{-dimethylbenzoyl})-N-(2\text{-methyl-3,4-(ethylenedioxy)-}$  35  $\text{benzoyl})-N'-(\text{tert-butyl})$  hydrazine, 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, and the like.

090424060  
031699  
86970-83424060

Ligands contemplated for use in the practice of the present invention are characterized as not normally being present in the cells of the subject, meaning that the  
5 ligand is exogenous to the subject. Ecdysteroids, for example, are not naturally present in mammalian systems. Thus, in accordance with the invention method, unless and until an ecdysteroid is administered to the subject, substantially no expression of the desired gene occurs.

10

An effective amount of ligand contemplated for use in the practice of the present invention is the amount of ligand (i.e., ecdysteroid) required to achieve the desired level of gene expression product. Ligand can be  
15 administered in a variety of ways, as are well-known in the art. For example, such ligands can be administered topically, orally, intravenously, intraperitoneally, intravascularly, and the like.

20

As readily recognized by those of skill in the art, it may be desirable to be able to rapidly induce or rapidly turn off expression by the invention expression system. This can readily be accomplished by administration of a suitable ecdysone antagonist before or after induction  
25 of the system (e.g., to prevent undesired activation of the system, to promote rapid induction, to rapidly terminate expression, and the like). Numerous ecdysone antagonists are known in the art, e.g., ajugalactone.

30

In accordance with a particular embodiment of the present invention, pharmaceutically acceptable formulations, and kits thereof, comprising at least one ecdysteroid, and a pharmaceutically acceptable carrier are contemplated. In accordance with another aspect of the  
35 present invention, pharmaceutically acceptable formulations consisting essentially of at least one ecdysteroid and a pharmaceutically acceptable carrier, are contemplated.

09042433-031698

Pharmaceutical formulations of the present invention can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more of the  
5 ecdysteroids of the present invention, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications.

10           The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers suitable for oral, topical, nasal, transdermal, intravenous, subcutaneous, intramuscular, intracutaneous, intraperitoneally, intravascular and the  
15 like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, and the like, is contemplated. Exemplary pharmaceutically acceptable carriers include  
20 carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. Such carriers which can be used include glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin,  
25 colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used.  
30 The active compound (i.e., ecdysteroid as described herein) is included in the pharmaceutically acceptable formulation in an amount sufficient to produce the desired effect upon the process or condition of diseases.

35           Pharmaceutically acceptable formulations containing the active ingredient may be in a form suitable for oral use, for example, as aqueous or oily suspensions,

09042493 031593

syrups or elixirs, tablets, troches, lozenges, dispersible  
 powders or granules, emulsions, or hard or soft capsules.  
 For the preparation of oral liquids, suitable carriers  
 include emulsions, solutions, suspensions, syrups, and the  
 5 like, optionally containing additives such as wetting  
 agents, emulsifying and suspending agents, dispersing  
 agents, sweetening, flavoring, coloring, preserving and  
 perfuming agents, and the like. Formulations intended for  
 oral use may be prepared according to any method known to  
 10 the art for the manufacture of pharmaceutically acceptable  
 formulations.

Tablets containing the active ingredient in  
 admixture with non-toxic pharmaceutically acceptable  
 15 excipients may also be manufactured by known methods. The  
 excipients used may be, for example, (1) inert diluents  
 such as calcium carbonate, lactose, calcium phosphate or  
 sodium phosphate; (2) granulating and disintegrating agents  
 such as corn starch, potato starch or alginic acid;  
 20 (3) binding agents such as gum tragacanth, corn starch,  
 gelatin or acacia, and (4) lubricating agents such as  
 magnesium stearate, stearic acid or talc. The tablets may  
 be uncoated or they may be coated by known techniques to  
 delay disintegration and absorption in the gastrointestinal  
 25 tract and thereby provide a sustained action over a longer  
 period. For example, a time delay material such as  
 glyceryl monostearate or glyceryl distearate may be  
 employed. They may also be coated by the techniques  
 described in the U.S. Pat. Nos. 4,256,108; 4,160,452; and  
 30 4,265,874, to form osmotic therapeutic tablets for  
 controlled release.

In some cases, formulations for oral use may be  
 in the form of hard gelatin capsules wherein the active  
 35 ingredient is mixed with an inert solid diluent, for  
 example, calcium carbonate, calcium phosphate or kaolin.  
 They may also be in the form of soft gelatin capsules

RECORDED " 88424060

wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

5           The pharmaceutically acceptable formulations may be in the form of a sterile injectable suspension. Suitable carriers include non-toxic parenterally-acceptable sterile aqueous or non-aqueous solutions, suspensions, or emulsions. This suspension may be formulated according to  
10 known methods using suitable dispersing or wetting agents and suspending agents. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use. Sterile, fixed oils are conventionally employed as a solvent or suspending medium.  
15 For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate or the  
20 like. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the formulations, by irradiating the formulations, or by heating the formulations. Sterile injectable suspensions may also contain adjuvants such as  
25 preserving, wetting, emulsifying, and dispersing agents. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Compounds contemplated for use in the practice of  
30 the present invention may also be administered in the form of suppositories for rectal administration of the drug. These formulations may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters of polyethylene glycols, which  
35 are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the drug.

09042488-031698

The pharmaceutically acceptable formulations are administered in a manner compatible with the route of administration, the dosage formulation, and in a therapeutically effective amount. The required dosage will vary with the particular treatment desired, the degree and duration of therapeutic effect desired, the judgment of the practitioner, as well as properties peculiar to each individual. Moreover, suitable dosage ranges for systemic application depend on the route of administration. It is anticipated that dosages between about 10 micrograms and about 1 milligram per kilogram of body weight per day will be used for therapeutic treatment.

An effective amount of the pharmaceutically acceptable formulation contemplated for use in the practice of the present invention is the amount of the pharmaceutically acceptable formulation (e.g., ecdysteroids(s)) required to achieve the desired level of transcription and/or translation of exogenous nucleic acid. A therapeutically effective amount is typically an amount of a ligand or ligand precursor that, when administered in a pharmaceutically acceptable formulation, is sufficient to achieve a plasma concentration of the transcribed or expressed nucleic acid product from about 0.1  $\mu\text{g/ml}$  to about 100  $\mu\text{g/ml}$ , preferably from about 1.0  $\mu\text{g/ml}$  to about 50  $\mu\text{g/ml}$ , more preferably at least about 2  $\mu\text{g/ml}$  and usually 5 to 10  $\mu\text{g/ml}$ .

Pharmaceutically acceptable formulations containing suitable ligand(s) are preferably administered intravenously, as by injection of a unit dose, for example.

The term "unit dose," when used in reference to a pharmaceutically acceptable formulation of the present invention, refers to a quantity of the pharmaceutical formulation suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active

09042433-031699B

material calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle. It may be particularly advantageous to administer such formulations in depot or long-lasting 5 form as discussed hereinafter.

Suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more 10 intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

15 Ecdysone response elements contemplated for use in the practice of the present invention (relating to modulation of the expression of exogenous genes in a subject) include native, as well as modified ecdysone 20 response elements. Since invention modified ecdysone receptors can function as either homodimers or as heterodimers (with a silent partner therefor), any response element that is responsive to an invention modified ecdysone receptor, in the form of a homodimer or 25 heterodimer, is contemplated for use in the invention methods described herein. As is readily recognized by those of skill in the art, modified receptors according to the invention (whether in the form of a homodimer or a heterodimer) can bind to either a response element having 30 an inverted repeat motif (i.e., two or more half sites in mirror image orientation with respect to one another), or to a response element having a direct repeat motif.

In a preferred embodiment of the invention, 35 invention modified ecdysone response elements are engineered so as to no longer be capable of binding to a farnesoid hormone receptor (since the mammalian farnesoid

09042488-031698

hormone receptor is able to bind to native ecdysone receptor response element). Invention modified ecdysone response elements provide low background expression levels of the exogenous gene and increase the selectivity of the gene expression system when used in mammalian systems.

Ecdysone response elements contemplated for use herein are short cis-acting sequences (i.e., having about 12-20 bp) that are required for activation of transcription in response to a suitable ligand, such as ecdysone or muristerone A, associated with a particular hormone receptor. The association of these response elements with otherwise ecdysone-nonresponsive regulatory sequences causes such regulatory sequences to become ecdysone responsive. Ecdysone response element sequences function in a position- and orientation-independent fashion.

The native ecdysone response element has been previously described, see, e.g., Yao et al., Cell, 71:63-72, 1992. Modified ecdysone response elements according to present invention comprise two half-sites (in either direct repeat or inverted repeat orientation to one another), separated by a spacer of 0-5 nucleotides. As used herein, the term "half-site" refers to a contiguous 6 nucleotide sequence that is bound by a particular member of the steroid/thyroid hormone superfamily of receptors. Each half-site is typically separated by a spacer of 0 up to about 5 nucleotides. Typically, two half-sites with a corresponding spacer make up a hormone response element. Hormone response elements can be incorporated in multiple copies into various transcription regulatory regions.

Preferred modified ecdysone response elements according to the invention comprise, in any order, a first half-site and a second half-site separated by a spacer of 0-5 nucleotides;

090424060 03424060

wherein the first and second half-sites are inverted with respect to each other;

wherein said first half-site has the sequence:

5 -RGBNNM-,

(or complements thereof) wherein

each R is independently selected from A or G;

each B is independently selected from G, C, or T;

each N is independently selected from A, T, C, or

10 G; and

each M is independently selected from A or C;

with the proviso that at least 4 nucleotides of each -RGBNNM- group of nucleotides are identical with the nucleotides at comparable positions of the sequence

15 -AGGTCA-; and

said second half-site is obtained from a glucocorticoid receptor subfamily response element.

The complement to the -RGBNNM- sequence set forth  
20 above is:

-YCVNNK-,

wherein

each Y is independently selected from T or C;

each V is independently selected from C, G, or A;

25 each N is independently selected from A, T, C, or G; and

each K is independently selected from T or G.

Exemplary first half-sites having the -RGBNNM-  
30 motif for use in the invention modified ecdysone response element include, for example, half-sites selected from -AGGGCA-, -AGTTCA-, -AGGTAA-, -AGGTCA-, -GGTTCA-, -GGGTTA-, -GGGTGA-, -AGGTGA-, or -GGGTCA-. A particularly preferred first half-site is -AGTGCA-.

35

Glucocorticoid receptor subfamily response elements contemplated for use in the practice of the

09042498-031698

present invention are response elements having half-sites that are typically bound by glucocorticoid, mineralocorticoid, progesterone or androgen receptors. Suitable half-sites from glucocorticoid receptor subfamily  
 5 response elements can be selected from the following sequence (in either orientation):

-RGNNCA-

(or complements thereof such as -YCNNGT-), wherein R, Y and N are as defined above. Exemplary half-sites having the  
 10 -RGNNCA- motif for use in the invention modified ecdysone response element include -AGAACA-, -GGAACA-, -AGTTCA-, -AGGTCA-, -GGAACA-, -GGTTCA-, -GGGTCA-, -GGGTCA-, -AGGTGA-, -GGGTCA-, and the like, as well as complements thereof. Particularly preferred half-sites having the  
 15 -RGNNCA- motif include -AGAACA- and -GGAACA-, with -AGAACA- being especially preferred.

When the above-described modified ecdysone response elements are employed to bind invention  
 20 heterodimeric receptors, the second half-site is inverted with respect to the first half-site. For example, when describing a single-strand of an invention modified ecdysone response element in the 5'-3' direction, the following general motif can be employed:

25

RGBNNM-(N)<sub>x</sub>-TGNNCY (SEQ ID NO:10),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred. As an alternative orientation to the  
 30 above described response element motif (SEQ ID NO:10), an invention response element can be described in the 5'-3' direction as:

35

RGNNCA-(N)<sub>x</sub>-KNNVCY (SEQ ID NO:11),

where x is an integer of 0 up to about 5, with x = 1 being especially preferred.

09042488-031698

In preferred embodiments of the present invention, the first half-site is obtained from an ecdysone response element and the second half-site is obtained from  
 5 a hormone response element selected from a glucocorticoid response element, a mineralocorticoid response element, a progesterone response element or an androgen response element. In a particularly preferred embodiment of the present invention, the first half-site is obtained from an  
 10 ecdysone response element and the second half-site is obtained from a glucocorticoid response element.

In a particularly preferred embodiment of the invention modified ecdysone response element, the first  
 15 half-site is AGTGCA and said second half-site is TGTCT. The presently most preferred modified-ecdysone response element for use in the invention methods is:

AGTGCA-N-TGTCT (SEQ ID NO:12).

20

In another aspect of the invention, when modified ecdysone receptors of the invention exist as homodimers, response elements employed preferably have a direct repeat motif (instead of the above-described inverted repeat  
 25 motif), as follows:

RGBNNM-(N)<sub>x'</sub>-RGBNNM (SEQ ID NO:13),

where R, B, N and M are as previously defined, and x' is an  
 30 integer of 0 up to about 5, with x' = 3 being especially preferred.

Invention modified ecdysone response elements are characterized as having substantially no constitutive  
 35 activity, which refers to the substantial absence of background levels of gene expression initiated by invention modified ecdysone response elements when introduced into

05042458-031698

mammalian expression systems. Since it has been found that mammalian farnesoid hormone receptors are able to bind to and transactivate gene expression from native ecdysone response elements, in certain embodiments of the present invention (e.g., where it is desired to avoid farnesoid-mediated background expression), modified ecdysone response elements are employed.

Presently preferred invention modified ecdysone response elements are further characterized as having substantially no binding affinity for farnesoid X receptor (FXR), i.e., invention response elements are incapable of binding FXR (which would thereby create undesired background levels of expression). Thus, presently preferred invention modified ecdysone response elements preferably induce basal levels of expression of substantially zero.

Response elements employed in the practice of the present invention are operably linked to a suitable promoter for expression of exogenous gene product(s). As used herein, the term "promoter" refers to a specific nucleotide sequence recognized by RNA polymerase, the enzyme that initiates RNA synthesis. This sequence is the site at which transcription can be specifically initiated under proper conditions. When exogenous genes, operatively linked to a suitable promoter, are introduced into the cells of a suitable host, expression of the exogenous genes is controlled by the presence of ecdysteroid compounds, which are not normally present in the host cells.

In accordance with another embodiment of the present invention, there are provided methods of inducing the expression of an exogenous gene in a mammalian subject containing:

09042438-031698

- (i) a DNA construct comprising an exogenous gene under the control of an ecdysone response element,
- (ii) DNA encoding a modified ecdysone receptor under the control of an inducible promoter; wherein said modified ecdysone receptor, in the presence of a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, and
- (iii) a ligand for said modified ecdysone receptor;
- said method comprising subjecting said subject to conditions suitable to induce expression of said modified ecdysone receptor.

Inducible promoters contemplated for use in the practice of the present invention are transcription regulatory regions that do not function to transcribe mRNA unless inducing conditions are present. Examples of suitable inducible promoters include DNA sequences corresponding to: the *E. coli* lac operator responsive to IPTG (see Nakamura et al., Cell, 18:1109-1117, 1979); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g. zinc) induction (see Evans et. al, U.S. Patent No. 4,870,009), the phage T7lac promoter responsive to IPTG (see Studier et al., Meth. Enzymol., 185: 60-89, 1990; and U.S. #4,952,496), the heat-shock promoter, and the like.

In accordance with another embodiment of the present invention, there are provided methods of inducing expression of an exogenous gene in a mammalian subject containing a DNA construct comprising said exogenous gene

00442438-031698

under the control of an ecdysone response element, said method comprising introducing into said subject:

a modified ecdysone receptor; and

a ligand for said modified ecdysone  
5 receptor,

wherein said receptor, in combination with a ligand therefor, and optionally in the further presence of a receptor capable of acting as a silent partner therefor, binds to said ecdysone response element, activating  
10 transcription therefrom.

In accordance with another embodiment of the present invention, there are provided methods for the expression of recombinant products detrimental to a host  
15 organism, said method comprising:

transforming suitable host cells with:

- (i) a DNA construct encoding said recombinant product under the control of an ecdysone response element, and
- 20 (ii) DNA encoding a modified ecdysone receptor;

growing said host cells in suitable media; and inducing expression of said recombinant product by introducing into said host cells ligand(s) for said  
25 modified ecdysone receptor, and optionally a receptor capable of acting as a silent partner for said modified ecdysone receptor.

Recombinant products detrimental to a host  
30 organism contemplated for expression in accordance with the present invention include any gene product that functions to confer a toxic effect on the organism. For example, inducible expression of a toxin such as the diptheroid toxin would allow for inducible tissue specific ablation  
35 (Ross et al. (1993) *Genes and Development* 7, 1318-1324). Thus, the numerous gene products that are known to induce apoptosis in cells expressing such products are

031699-034433-034433

5

10

20

30

35

vectors typically come in the form of a plasmid that, upon introduction into an appropriate host cell, results in expression of the inserted DNA.

5           As used herein, the phrase "transcription regulatory region" refers to the region of a gene or expression construct that controls the initiation of mRNA transcription. Regulatory regions contemplated for use herein typically comprise at least a minimal promoter in  
10 combination with an ecdysone response element. A minimal promoter, when combined with an enhancer region (e.g., a hormone response element), functions to initiate mRNA transcription in response to a ligand/receptor complex. However, transcription will not occur unless the required  
15 inducer (ligand) is present.

As used herein, the phrase "operatively associated with" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides,  
20 such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and promoter such that the transcription of such DNA is  
25 initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

Preferably, the transcription regulatory region further comprises a binding site for an ubiquitous  
30 transcription factor. Such a binding site is preferably positioned between the promoter and modified ecdysone response element of the invention. Suitable ubiquitous transcription factors for use herein are well-known in the art and include, for example, Sp1.

35

Expression vectors suitable for use in the practice of the present invention are well known to those

09042488-0316998

of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells as well as those that remain episomal and those that integrate into the host cell genome. Expression vectors typically further  
 5 contain other functionally important nucleic acid sequences, such as expression constructs encoding antibiotic resistance proteins, and the like.

Exemplary eukaryotic expression vectors include  
 10 eukaryotic constructs, such as the pSV-2 gpt system (Mulligan et al., Nature, 1979, 277:108-114); pBlueSkript (Stratagene, La Jolla, CA), the expression cloning vector described by Genetics Institute (Science, 1985, 228:810-815), and the like. Each of these plasmid vectors are  
 15 capable of promoting expression of the invention chimeric protein of interest.

Promoters, depending upon the nature of the regulation, may be constitutively or inducibly regulated,  
 20 or may be tissue-specific (e.g., expressed only in T-cells, endothelial cells, smooth muscle cells, and the like). Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor  
 25 virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoter, albumin promoter, APO A1 promoter, cyclic AMP dependent kinase II (CaMKII) promoter, keratin promoter, CD3 promoter, immunoglobulin light or heavy chain  
 30 promoters, neurofilament promoter, neuron specific enolase promoter, L7 promoter, CD2 promoter, myosin light chain kinase promoter, HOX gene promoter, thymidine kinase (TK) promoter, RNA Pol II promoter, MYOD promoter, MYF5 promoter, phosphoglycerokinase (PGK) promoter, Stf1  
 35 promoter, Low Density Lipoprotein (LDL) promoter, and the like.

09042488-031698

Suitable means for introducing (transducing) expression vectors containing nucleic acid constructs according to the invention into host cells to produce 5 transduced recombinant cells (i.e., cells containing recombinant heterologous nucleic acid) are well-known in the art (see, for review, Friedmann, 1989, Science, 244:1275-1281; Mulligan, 1993, Science, 260:926-932, each of which are incorporated herein by reference in their 10 entirety). Exemplary methods of transduction include, e.g., infection employing viral vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), calcium phosphate transfection (U.S. Patents 4,399,216 and 4,634,665), dextran sulfate transfection, electroporation, lipofection 15 (see, e.g., U.S. Patents 4,394,448 and 4,619,794), cytofection, particle bead bombardment, and the like. The heterologous nucleic acid can optionally include sequences which allow for its extrachromosomal (i.e., episomal) maintenance, or the heterologous nucleic acid can be donor 20 nucleic acid that integrates into the genome of the host.

In a specific embodiment, said gene transfer vector is a viral vector, preferably a retroviral vector. Retroviral vectors are gene transfer plasmids that have an 25 expression construct encoding an heterologous gene residing between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in 30 an appropriate packaging cell line (see, e.g., U.S. Patent 4,650,764).

Suitable retroviral vectors for use herein are described, for example, in U.S. Patents 5,399,346 and 35 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, incorporated herein by reference, which provide a

09042488-031698

description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e.g., Shackelford et al., 5 1988, PNAS, USA, 85:9655-9659), and the like.

Various procedures are also well-known in the art for providing helper cells which produce retroviral vector particles which are essentially free of replicating virus.

10 See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy, 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988);  
15 and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806 (1987), which disclose procedures for producing viral vectors and helper cells which minimize the chances for producing a viral vector which includes a replicating virus.

20  
Recombinant retroviruses suitable for carrying out the invention methods are produced employing well-known methods for producing retroviral virions. See, for example, U.S. Patent 4,650,764; Miller, Human Gene Therapy,  
25 1:5-14 (1990); Markowitz, et al., Journal of Virology, 61(4):1120-1124 (1988); Watanabe, et al., Molecular and Cellular Biology, 3(12):2241-2249 (1983); Danos, et al., Proc. Natl. Acad. Sci., 85:6460-6464 (1988); and Bosselman, et al., Molecular and Cellular Biology, 7(5):1797-1806  
30 (1987).

In accordance with another embodiment of the present invention, there are provided recombinant cells containing a nucleic acid encoding modified ecdysone  
35 receptor(s) according to the invention. Exemplary eukaryotic cells for introducing expression vectors according to the invention include, e.g., CV-1 cells, P19

0904248-031609

cells and NT2/D1 cells (which are derived from human embryo carcinomas), COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, primary human fibroblast cells, human embryonic kidney cells, African green monkey cells, HEK 293 5 (ATCC accession #CRL 1573; U.S. Patent No. 5,024,939), Ltk<sup>-</sup> cells (ATCC accession #CCL1.3), COS-7 cells (ATCC under accession #CRL 1651), DG44 cells (dhfr<sup>-</sup> CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555), cultured primary tissues, cultured tumor cells, and the 10 like. Presently preferred cells include CV-1 and 293 cells.

In accordance with another embodiment of the present invention, there is provided a transgenic mammal 15 containing a nucleic acid encoding an invention modified ecdysone receptor. As used herein, the phrase "transgenic mammal" refers to a mammal that contains one or more inheritable expression constructs containing a recombinant modified ecdysone receptor transgene and/or an exogenous 20 gene under the transcription control of an invention modified ecdysone response element. Preferably, an invention transgenic mammal also contains one or more inheritable expression constructs containing a member of the steroid/thyroid hormone superfamily of receptors that 25 functions as a silent partner for modified ecdysone receptor (e.g., RXR).

Methods of making transgenic mammals using a particular nucleic acid construct are well-known in the 30 art. When preparing invention transgenic animals, it is preferred that two transgenic lines are generated. The first line will express, for example, RXR and a modified EcR (e.g., VpEcR). Tissue specificity is conferred by the selection of tissue-specific promoters (e.g., T-cell 35 specific) that will then direct the expression of the receptors. A second line contains an ecdysone responsive promoter controlling the expression of an exogenous cDNA.

031693 031693 031693

In a preferred embodiment of the present invention, an invention transgenic mammal contains one or more expression constructs containing nucleic acid encoding  
5 a modified ecdysone receptor, exogenous RXR, and an exogenous gene under the transcription control of an invention modified ecdysone response element. It has been found that in transgenic mice containing an ecdysone inducible promoter (i.e., an invention modified ecdysone  
10 response element) and expressing a modified ecdysone receptor and RXR, muristerone treatment can activate gene expression. Thus, with tissue specific expression of the modified ecdysone receptor and RXR and timely hormone treatment, inducible gene expression can be achieved with  
15 spatial, dosage, and temporal specificity.

In accordance with another embodiment of the present invention, there are provided methods for inducing expression of an exogenous gene in a transgenic mammal  
20 containing a modified ecdysone receptor according to the invention, said method comprising:

introducing into said mammal a DNA construct encoding an exogenous gene under the transcription control of an ecdysone response  
25 element responsive to said modified ecdysone receptor; and

administering to said mammal an amount of ligand for said modified ecdysone receptor effective to induce expression of said exogenous  
30 gene.

As discussed hereinbefore, the modified ecdysone receptor forms a homodimer, or optionally a heterodimer in the presence of a silent partner of the steroid/thyroid hormone  
35 superfamily of receptors, and functions to activate transcription from an expression vector having a response

05042438-031699B

element responsive to the particular homodimer or heterodimer formed.

In accordance with another embodiment of the present invention, there are provided methods for the induction of two different genes in a mammalian subject comprising: activating a first exogenous gene employing the invention ecdysone inducible system; and activating a second gene using a tetracycline inducible system. The invention method for inducing two different genes is particularly advantageous because it permits the temporal, spatial, and dosage specific control of two exogenous genes.

The tetracycline inducible system is well-known in the art (see, e.g, Gossen et al. (1992) *Proc. Natl. Acad. Sci.* **89**, 5547-5551; Gossen et al.(1993) *TIBS* **18**, 471-475; Furth et al. (1994) *Proc. Natl. Acad. Sci.* **91**, 9302-9306; and Shockett et al. (1995) *Proc. Natl. Acad. Sci.* **92**, 6522-6526).

All U.S. and Foreign Patent publications, textbooks, and journal publications referred to herein are hereby expressly incorporated by reference in their entirety. The invention will now be described in greater detail by reference to the following non-limiting examples.

#### Example 1

##### Preparation of modified ecdysone receptors

30

##### Plasmid preparation:

The plasmids CMX-EcR, CMX-USP, CMX-FXR, CMX-hRXRa, EcREx5- $\Delta$ MTV-Luc, CMX-GEcR, MMTV-luc, and CMX-GR have been previously described (Yao, et al., *Nature* **366**:476-479 (1993) and Forman, et al. *Cell* **81**:687-693 (1995)).

004424060

The plasmid CMX-VpEcR was constructed by ligation of an EcoRI fragment of psk-EcR and CMX-Vp16.

5           The plasmid CMX-VgEcR was generated by site-directed mutagenesis of CMX-VpEcR using the Transformer Mutagenesis Kit (Clontech) and the mutagenic Oligonucleotide (SEQ ID NO:14):

10 5'-TACAACGCCCTCACCTGTGGATCCTGCAAGGTGTTTCTTTTCGACGCAGC-3'.

Mutagenesis of VpEcR to VgEcR altered the P-box region of the DNA binding domain of ecdysone receptor to resemble that of GR (Umesono and Evans, *Cell* 57:1139-1146 (1989)).

15 The following amino acids in the DNA-binding domain of the ecdysone receptor were altered: E282G, G283S, and G286V (E=glutamate, G=glycine, S=serine, V=valine).

The reporter construct EcREx4- $\Delta$ HSP- $\beta$ -gal was  
20 constructed by oligomerizing two annealed oligonucleotides containing the HSP-EcRE (Yao, et al., *Nature* 366:476-479 (1993)).

EcREx4-Sp1x3- $\Delta$ HSP- $\beta$ gal was constructed by  
25 ligating the following annealed oligonucleotides into the Asp718 site of EcREx4-HSP- $\beta$ -gal (SEQ ID NO:15):

5'-GTACTCCCGGGGCGGGGCTATGCGGGGCGGGGCTAATCGCTAGGGGCGGGGCA-3'

30 and (SEQ ID NO:16):

5'-GTACTGCCCCGCCCCTAGCGATTAGCCCCGCCCCGCATAGCCCCGCCC  
CGGGA-3'.

35  $\Delta$ HSP is a minimal promoter derived from the *Drosophila* heat shock promoter with its enhancers deleted.

050424-88-031698

5 5'-AGCTCGATGGACAAGTGCATTGTTCTTTGCTGAA-3';

5'-AGCTTTCAGCAAGAGAACAATGCACTTGTCCATCG-3',  
10

15

20

## 25

30

Transfections using luciferase as the reporter were assayed by standard techniques using luciferin and ATP. These values were normalized by co-transfection of CMX- $\beta$ -galactosidase. Hormone treated cells were treated with ethanol, 50  $\mu$ M Juvenile Hormone III (Sigma), 1 $\mu$ M

muristerone A (Zambon, Bresso, IT), or 1 $\mu$ M dexamethasone (Sigma) unless otherwise noted.

To maximize the sensitivity of the invention  
 5 ecdysone inducible system, modifications of the ecdysone receptor were made. The N-terminal transactivation domain of the ecdysone receptor was replaced by the corresponding domain of the glucocorticoid receptor (GR), resulting in the modified ecdysone receptor GEcR (See Figure 1D). CV-1  
 10 cells were transfected with the plasmid CMX-GEcR encoding the modified ecdysone receptor as discussed above. After transfection, cells were either treated with ethanol or 1 $\mu$ M muristerone A. This hybrid modified ecdysone receptor boosted muristerone responsiveness from 3- to 11-fold in a  
 15 transient transfection assay (Fig. 1A). Replacement of the natural heterodimeric partner for the ecdysone receptor, USP, by its mammalian homologue, the retinoid X receptor (RXR), produced a more potent ligand dependent heterodimer, providing a 34 fold induction (Fig. 1A).

20 A more potent heterodimer, however, was obtained by combining RXR and VpEcR, an N-terminal truncation of the ecdysone receptor attached to the VP16 activation domain, resulting in a 212 fold induction (Fig. 1A and 1D).  
 25 Different from most nuclear receptor/VP16 fusion proteins which exhibit high constitutive activity, VpEcR generates ligand dependent superinduction while maintaining a very low basal activity (Underhill et al., *Mol. Encod.* 8:274-285 (1994) and Perlmann et al., *Genes & Devel.* 7:1411-1422  
 30 (1993)).

In addition, the reporter vector was also modified by inserting consensus binding sites for the ubiquitous transcription factor Sp1 between the minimal  
 35 promoter and the ecdysone response elements (Kamine et al., *Proc. Natl. Acad. Sci.* 88:8510-8514 (1991) and Strahle et

al., *EMBO* 7:3389-3395 (1988)). The addition of Sp1 sites to the ecdysone responsive promoter increases the absolute activity 5-fold (Fig. 1A).

5

## Example 2

### Construction of a novel ecdysone response element

Although no mammalian transcription factors have been shown to have a natural enhancer element like the  
10 ecdysone response element, which is composed of two inverted half-sites of the sequence AGGTCA spaced by one nucleotide, it is difficult to preclude such a possibility.

The recently cloned farnesoid X receptor (FXR) can very weakly activate certain synthetic promoters containing an  
15 ecdysone response element in response to extremely high concentrations of farnesoids (Forman et al., *Cell* 81:687-693 (1995)).

In FXR containing cells and in transgenic mice,  
20 activation of gene expression by endogenous receptors would create undesirable background levels of reporter protein. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR, which binds as a homodimer to an inverted repeat of the sequence  
25 AGAACA, spaced by three nucleotides. This altered binding specificity was achieved by mutating three amino acid residues of VpEcR in the P-box of the DNA binding domain, a region previously shown to be essential for DNA sequence recognition (Umesono and Evans, *Cell* 57:1139-1146 (1989)).  
30 This new hybrid modified ecdysone receptor is referred herein as VgEcR and is responsive to a new hybrid response element referred to herein as the E/GRE (SEQ ID NO:12), which contains two different half-site motifs, RGBNNM and RGNNCA, spaced by one nucleotide (Fig. 1B). This new  
35 response element is a hybrid between the glucocorticoid response element (GRE) and that of type II nuclear receptors like RXR, EcR, retinoic acid receptor (RAR),

09042493-031698

thyroid hormone receptor (T3R), etc. Although FXR can activate a promoter containing the wild type ecdysone response element, it cannot activate one containing the E/GRE (Fig 1B; note log scale). The E/GRE reporter is not  
 5 activated by GR nor does VgEcR activate a dexamethasone responsive promoter (Fig 1C).

### Example 3

#### Assay for Ecdysone responsiveness in stable cell lines

10

Stable cell lines were generated containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and an ecdysone inducible reporter (Figure 2). 293 cells were transfected with the following linearized  
 15 plasmids, pRC-ESH $\beta$ , EcREx5- $\Delta$ MTV-Luc, CMX-VpEcR, and CMX-hRXRa. The following day, the cells were split 1:10 and were allowed to recover one day prior to selection with 1mg/ml G418 (GIBCO). After 14 days of selection, 14 individual clones were isolated and grown separately in the  
 20 presence of 0.5mg/ml G418. Of 14 G418 resistant clones, 10 demonstrated differing degrees of muristerone responsiveness.

One of these cell lines, N13, was grown in the  
 25 presence or absence of 1 $\mu$ M muristerone for 20 hours. Cell lysates were then assayed for  $\beta$ -galactosidase and luciferase activities as described in Example 1. X-gal staining was performed on the stable cell lines. Cells were fixed briefly with 10% formaldehyde in PBS and then  
 30 stained with X-Gal (Molecular Probes, Eugene, OR) for 2 to 6 hours. After 24 hours of treatment with 1 $\mu$ M muristerone, 100% of the cells turned dark blue after 3 hours of staining. Thus, mammalian cells containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and  
 35 a reporter gene construct regulated by a modified ecdysone

0042468-031698

response element, function to efficiently express an exogenous gene in response to a ligand, e.g., ecdysone.

A dose-response assay was conducted by growing 5 N13 cells with varying concentrations of muristerone for 36 hours and then assaying for  $\beta$ -galactosidase activity (using the well-known ONPG assay), or the cells were assayed for luciferase activity. Dose response curves of stably integrated  $\beta$ -galactosidase and luciferase reporters in N13 10 cells revealed that inducibility approaching 3 orders of magnitude can be achieved at a final concentration  $10\mu\text{M}$  muristerone (Figure 3A). One-hundred fold induction was achieved by muristerone concentrations as low as  $100\text{nM}$  (Figure 3A).

15 Finally, the kinetics of muristerone mediated induction was measured. N13 cells were grown in separate wells in the presence of  $1\mu\text{M}$  muristerone, harvested at varying times, and assayed for luciferase activity. 20 Inductions of 100-fold in 3 hrs., 1000 fold in 8 hrs., and maximal effects of 20,000 fold after 20 hours of treatment were observed (Figure 3B). Similar results were observed in stable lines containing CMX-VgEcR and the E/GRE reporters.

25

#### Example 4

##### Bioavailability and activity of muristerone

In order to use muristerone as a potential 30 hormone in mice, its toxicity and bioavailability was examined. For toxicity studies, adult mice were injected intraperitoneally with 20mg of muristerone A suspended in sesame oil. The mice were then observed for approximately two months. For teratogenic studies, pregnant mice were 35 injected with 20mg of muristerone A suspended in sesame oil and both the mother and pups were observed for three

09042498-031698

months. The results indicate that muristerone maintains its activity when injected into mice, and that it is neither toxic, teratogenic, nor inactivated by serum binding proteins. In addition to the inert qualities of muristerone (an ecdysone), overexpression of VpEcR and RXR appears not to be toxic.

For muristerone bioavailability studies, adult mice were injected intraperitoneally with sesame oil with or without 10mg of muristerone, and were subsequently sacrificed for serum collection. After twelve hours, blood was drawn from the mice, and the serum was isolated by brief centrifugation of the whole blood. In order to conduct transfection assays to test for muristerone activity, serum from sesame oil injected mice was divided, and half was supplemented with muristerone to a final concentration of 10 $\mu$ M. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRa, and EcREx5-DMTV-Luc.

The results are shown in Figure 4 and indicate that serum from muristerone treated mice yielded a luciferase activity comparable to that seen from untreated mouse serum supplemented with 1 $\mu$ M muristerone. The results indicate that single-site injected material should be widely circulated, and that there is little or no blunting of activity due to association with serum proteins.

#### Example 5

#### Muristerone dependent gene expression in transgenic mice

To produce transgenic mice, the following DNA constructs were prepared and subsequently injected into fertilized eggs: CD3-VpEcR, CD3-RXR, ESH $\beta$  (Lee et al., *J. Exp. Med.* 175:1013-1025 (1992)). Two separate lines of transgenic mice were generated harboring either an ecdysone

09042498-031698

inducible reporter, ESH $\beta$ , or a T-cell specific expression construct of VpEcR and RXR, respectively. The former are referred to as reporter mice, the latter are referred to as receptor mice, and double transgenic mice are referred to as receptor/reporter mice. Constructs CD3-VpEcR and CD3-RXR were mixed and coinjected, while ESH $\beta$  was injected alone. Primary genotyping was performed by Southern blot analysis and the transmission of transgenic mice was monitored by dot blot analysis. Receptor mice were analyzed for VpEcR and RXR expression by Northern blot analysis of RNA collected from these mice. For Northern blot analysis, 15 $\mu$ g of total RNA obtained from the thymus, and various tissues as a control, was run on a denaturing gel and blotted onto a nitrocellulose membrane. The blot was probed with a radiolabeled  $\beta$ -gal-specific probe and exposed on film for 2 days. These receptor mice were healthy, fertile, and appeared normal by visual inspection.

In addition, the transgene was transferred to the offspring as expected by Mendelian genetics. This data suggests that overexpression of VpEcR and RXR in T-cells is not toxic.

Receptor expressing mice were bred with reporter mice (containing ESH $\beta$ ) to produce double transgenic receptor/reporter mice. Adult receptor/reporter transgenic mice (genotype=CD3-VpEcR; CD3-RXR; and ESH $\beta$ ) were injected intraperitoneally with sesame oil with or without 10mg of muristerone. Subsequently, a Northern blot analysis was performed on the double transgenic lines using RNA isolated 48 hours after treatment from various tissues including the thymus, brain and liver, to test for the specific induction of an ecdysone inducible promoter. The probe used was specific to the activity of the ecdysone inducible promoter. The autoradiograph was exposed for 36 hrs. The results of the Northern analysis indicate that muristerone treatment of the transgenic mouse containing a T-cell

specific expression construct of VpEcR and RXR, and the ecdysone inducible reporter ESH $\beta$ , caused a significant induction from an ecdysone inducible promoter in the thymus, while low basal activity is observed in its absence.

#### Example 6

##### Assay for Ponasterone responsiveness

10           A dose-response assay was conducted as described in Example 3, by growing N13 cells with varying concentrations of muristerone or ponasterone A for 36 hours and then assaying for  $\beta$ -galactosidase activity (using the well-known ONPG assay), or the cells were assayed for  
15 luciferase activity. Dose response curves of stably integrated  $\beta$ -galactosidase and luciferase reporters in N13 cells revealed that inducibility exceeding 3 orders of magnitude can be achieved with both ligands at final concentrations of about  $10^{-4}$  (see Figure 5).

20           While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and  
25 claimed.

09042433-031693